2008 Annual Report

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Organization: Colorado State University
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Technical Report

A. Scientific and Technical Objectives

Conventional chemical-based synthesis of energetic materials uses toxic ingredients and produces many environmentally undesirable wastes. In addition, chemical-based production is not sustainable since many of the chemicals used in making these precursors are petroleum-based. An alternative to this is to produce precursors of energetic materials using a bio-based approach. Recently, significant progress has been made in identifying the genes and enzymes in some microbes that can produce precursors of energetic materials. This knowledge can now be exploited to use plants for producing precursors of energetic materials in a sustainable manner. The focus of our project is to use bacterial enzymes to engineer plant metabolism to produce precursors of energetic materials. The specific objectives of this proposal are:

1) Metabolic engineering of plants to produce high levels of phloroglucinol by introducing a bacterial gene that converts malonyl Co-A to phloroglucinol into plants.
2) Metabolic engineering of plants to produce butanetriol. This will be accomplished by introducing bacterial genes involved in butanetriol synthesis from xylose and arabinose.
3) To develop regeneration and robust stable transformation technologies for Miscanthus to introduce and express genes involved in synthesis of energetic materials.

B. Approach

Most plants naturally produce the precursors but not the enzymes to convert them to the desired precursors of energetic materials, whereas bacteria have the enzymes that convert these precursors into end product. Our approach is to express bacterial genes under constitutive and/or tissue-specific promoters to produce phloroglucinol and 1,2,4-butanetriol. The genes to be introduced are: xylose dehydrogenase, xylonate dehydratase, arabinose dehydrogenase, L-arabinose dehydrates, benzoylformate decarboxylase, dehydrogenase and *PhlD*. Because of many available resources for Arabidopsis, we will use this plant as a model system to test this concept. Once proven, these constructs will be introduced into Miscanthus, a non-food crop that is known to grow well on marginal soils with minimum or no inputs. Transformation of plants will be done using Agrobacterium-based disarmed binary vectors using floral-dip method (in case of Arabidopsis) and regeneration of plants in tissue culture after co-cultivation with Agrobacterium (in case of Miscanthus). Most of our initial work will be performed in Arabidopsis and the best gene constructs will be transferred to Miscanthus, a grass species. We will be using a sterile hybrid of Miscanthus for these studies. Propagation of this variety is done through rhizomes.

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C. Concise Accomplishments

This project was funded in April 2008 and the work began in the later part of April 08. The progress report presented here is for about 6 weeks. We have completed bioinformatics work on all the bacterial genes that we will be using in this work. We identified and obtained sequences of necessary bacterial genes, PhID (*Psuedomonas fluorescence*), D-xylose dehydrogenase (*Caulobactor crescentus*), D-xylonate dehydratase (*E. Coli*), L-Arabinose dehydrogenenase (*P.fluorescence*), L-arabinonate dehydratase (*Burkholderia vietnamiensis),* Benzoylformate decarboxylase *(P. putida*) and designed primers for amplification of these genes. We obtained these bacterial strains from ATCC, cultured them and isolated genomic DNA for cloning these genes. We obtained *Miscanthus* rhizomes and seedlings and grown them in greenhouse and established them in tissue culture for regeneration studies. For cloning the bacterial genes we have obtained all the necessary plant transformation vectors for constitutive and inducible expression of bacterial genes in plants.

D. Expanded Accomplishments

DNA from different bacterial strains that is being used for amplification of all the genes needed for the production of phloroglucinol and butanetriol is shown in Figure 1. Miscanthus plants that are being grown in greenhouse and established tissue culture are shown in Figure 2.

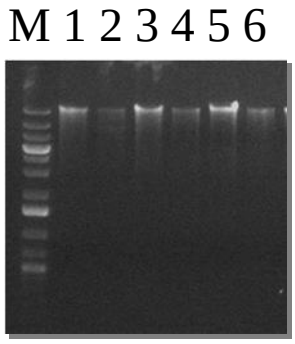

Figure 1. Genomic DNA from bacterial strains. M, DNA marker; Lanes 1,2. *P. putida;* Lanes 3,4. *P. fragi* and Lanes 5,6. *P.fluorescence*

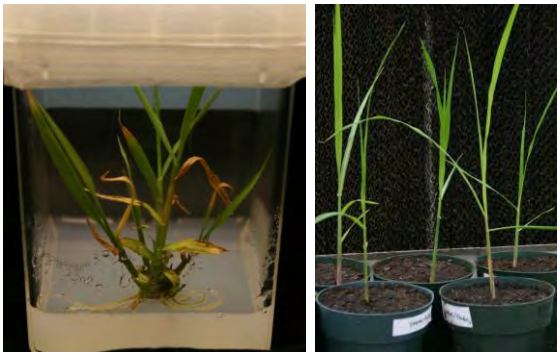

Figure. 2. *Miscanthus giganteus* plants growing in tissue culture (left) and in soil (right)

E. Work Plan:

1) Generation of transgenic Arabidopsis plants expressing bacterial PhlD gene

We will amplify *PhlD* by PCR using the genomic DNA from *Pseudomonas fluorescens* pf-5. We will verify the coding region by sequencing and insert it next to a constitutive promoter (Cauliflower mosaic virus 35S promoter, CaMV35S) in a plant transformation vector. The plasmid construct will be introduced into Agrobacterium GV3101 strain. Transformation of Arabidopsis will be performed by dipping flowering Arabidopsis plants in Agrobacterium culture. Transgenic plants will be selected using selection plates containing kanamycin. Several transformants will be selected and analyzed for the introduced genes by their expression level using RT-PCR and/or Northern analysis. The activity of PhlD in wild type and transgenic lines will be assayed by measuring the rate of NADH formation. Plants with high levels of mRNA expression and PhlD activity will be selfed to generate homozygous lines. Wild type and homozygous transgenic plants expressing varying levels of PhlD will be analyzed in the following year for the levels of phloroglucinol using GC-MS.

2) Generation of all constructs necessary for the production of butanetriol

Bacterial genes encoding six enzymes (D-xylose dehydrogenase, D-xylonate dehydratase, benzoylformate decarboxylase and dehydrogenase, L-arabinose dehydrogenase, L-arabinose dehydratase) are necessary for converting xylose and Arabinose to butanetriol. We will amplify these six genes, verify them by sequencing and clone them into vectors where each gene will be under a different constitutive promoter. We will then move four genes necessary to convert xylose to butanetriol into a single plant transformation vector. Similarly, all four genes needed to convert arabinose to butanetriol will be cloned into another plant transformation vector. We will then begin transformation studies with these constructs in Arabidopsis.

3) Establish plant regeneration conditions for Miscanthus

Different plant parts from two species of Miscanthus (*Miscanthus x giganteus,* a triploid species) will be used to develop regeneration methods. To develop a plant regeneration system, we will generate callus from different tissues (leaves, roots and rhizomes) from young seedlings and inflorescence. Segments of these tissues will be incubated in a tissue culture medium with varying ratios of plant hormones (primarily auxins and cytokinins, other hormones will also be used if necessary) to establish conditions that are optimum for generating callus. We will then use the calli generated from different tissues and alter hormonal conditions to induce shoots and roots.

F. Major Problems/Issues

We started this project very recently (about six weeks ago). We have not encountered any problems so far.

G. Technology Transfer

None. This project just started

H. Foreign Collaborators and Supported Foreign National
None